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Longitudinal Study of Plasma HIV-1 RNA Concentrations During the Asymptomatic Stage of HIV Infection Measured Using AMPLICOR HIV Monitor and NASBA HIV-1 RNA QT Tests

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The predictive value of two methods for measuring HIV RNA concentration in plasma was assessed in relation to CD4 lymphocyte counts during the asymptomatic period of infection. The design was a retrospective longitudinal case-control study for a mean period of 60 months involving 20 asymptomatic patients included in the French National prospective survey. The CD4 counts in these patients during the last 36 months of the study were stable (non-progressors) or declined (progressors).

Plasma RNA concentrations were determined in each subject annually using the AMPLICOR and NASBA techniques. Only AMPLICOR gave RNA titers above the cut-off value for all the patients. The techniques agreed satisfactorily, although there was a difference, median 0.4 $\log_{10}$, between the AMPLICOR and NASBA values.

The non-progressors had low and stable RNA concentrations. The concentration was higher in the progressors, according to the AMPLICOR technique, from their inclusion in the study, and according to the NASBA technique, from 1 year after inclusion. However, only four of ten individual progressors had stable plasma HIV RNA concentrations significantly above those of the non-progressors before the decline in their CD4 counts. These were all and only the patients with a decline in lymphocyte counts more than 100 CD4/mm^3/year. In each of the other progressors, the RNA concentration was not significantly different from those of the non-progressors.

Thus, when making decisions about therapy, plasma HIV RNA determinations cannot be used in place of CD4 counts and may provide valuable additional information. ***J. Med. Virol. 54:60–68, 1998.*** © 1998 Wiley-Liss, Inc.

KEY WORDS: HIV RNA; prognosis; asymptomatic infection; CD4 lymphocyte depletion

INTRODUCTION

Numerous markers have been investigated for their value for predicting the evolution of HIV infection to AIDS. To date, the best marker was the CD4 lymphocyte count. Several recent studies involving large cohorts of patients suggest that a high HIV RNA concentration in the plasma or within the lymphocytes at the early stage of infection is predictive of the onset of AIDS [Henrard et al., 1995; Mellors et al., 1995, 1996; O'Brien et al., 1996; Saksela et al., 1995; Wong et al., 1996]. This marker, whatever its value, appears to have long-term prognostic value, independent of the CD4 lymphocyte count. Mellors et al. [1996] reported that among patients with a CD4 lymphocyte count above 500 cells/mm^3, the risk of death within 10 years is 70% for those with RNA titers above 10,000 HIV RNA copies/ml, whereas it is 30% for those with titers below 10,000 HIV RNA copies/ml. These data are particularly helpful for therapeutic decisions concerning patients at very high risk of developing AIDS rapidly, but for the majority of patients do not help indicate the

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moment when anti-viral treatment should be started. The view that anti-viral treatment should be started so as to prevent the immunodeficiency caused by the virus is growing [Havlir and Richman, 1996]. As effective treatments are being made available, it may become possible in the near future to prevent the decline in the CD4 lymphocyte count. However, little is known about the duration of efficacy on the most recent treatments, and they may have toxic effects. Thus, it may be preferable not to treat patients with high and stable CD4 lymphocyte counts. A marker allowing prediction of the subsequent evolution of the CD4 lymphocyte count would therefore be beneficial.

The evolution of the concentration of HIV RNA in the plasma with time was investigated in relation to that of the CD4 lymphocyte count, in order to assess its value in therapeutic decision-making. The study was of a longitudinal case-control design, involving 20 patients followed for 5 years, with different CD4 lymphocyte counts, and who were asymptomatic on their inclusion in the French national prospective survey (SEROCO). The HIV RNA concentration was followed in both cases (the ten progressors) and controls (the ten non-progressors) using two currently commercially available techniques: AMPLICOR HIV Monitor test (AMPLICOR) and NASBA HIV-1 RNA QT test (NASBA). These two techniques are based on different approaches for RNA preparation, amplification, and measurement. They are faster and more reproducible than classic laboratory techniques and are appropriate for routine clinical practice. Three previous transversal studies have compared HIV RNA quantification by these techniques and found that their results agree [Lin et al., 1994; Revets et al., 1996; Vandamme et al., 1996]. A comparative analysis of the AMPLICOR HIV Monitor and NASBA HIV-1 RNA QT techniques is described for measuring HIV RNA in the plasma using a longitudinal case-control study.

PATIENTS AND METHODS

Patients

Twenty patients, subjects of the French national multicenter survey (SEROCO) [Bucquet et al., 1994; Carré et al., 1994], were studied retrospectively. SEROCO is a prospective survey which since 1988 has included 1,453 patients of which 180 are followed in our center (ACCTES). The inclusion criteria are the identification of seropositivity within a year or a known infection date. The protocol requires a clinical and biological examination every 6 months, or more frequently when there is clinical or biological evolution. The 20 patients included in this study were 17 men and three women, all contaminated by sexual contact. They consisted of ten non-progressors (controls) with stable or increasing CD4 lymphocyte counts (increase of two to 115 cells/mm^3/year), matched for lymphocyte count on inclusion, inclusion date (±6 months), and age (±5 years) with ten progressors (cases): patients with declining CD4 lymphocyte counts during the last 3 years of the study (decrease of −46 to −224 cells/mm^3/year). Five progressors, referred to as "intermediate progressors," presented moderate decreases (<100 cells/mm^3/year) in CD4 lymphocyte counts (range −46 to −90 cells/mm^3/year), and the remaining five presented substantial decreases (⩾100 cells/mm^3/year; range −100 to −204 cells/mm^3/year) and are referred to as "rapid progressors." The patients were followed for a median of 5 years (range 3–5 years). One sample was collected from each patient every year and analyzed (median 6, range 4–6 samples per patient). Thus, a total of 106 samples (53 from progressors, 53 from non-progressors) was studied.

On inclusion in the SEROCO study, the CD4 lymphocyte counts were between 200 and 400 cells/mm^3 for one case (progressor) control (non-progressor) pair, between 400 and 600 cells/mm^3 for three pairs and above 600 cells/mm^3 for six pairs. All patients were asymptomatic (stage A) [Center for Disease Control and Prevention, 1992] and were not receiving treatment on inclusion. At the end of the study, the CD4 lymphocyte counts of the non-progressors were between 270 and 927 cells/mm^3, whereas the lymphocytes counts for the progressors were between eight and 694 cells/mm^3. Twelve patients were stage A, five were stage B, and three were stage C. Six patients (all progressors) were receiving treatment comprising anti-retroviral monotherapy (zidovudine, two patients; didanosine, two patients) or bitherapy (zidovudine and didanosine, two patients).

Methods

HIV RNA genomes were titrated in plasma collected on sodium heparin using the NASBA HIV-1 RNA QT test (Organon Teknica, Boxtel, The Netherlands) [Van Gemen et al., 1994] and in serum using the AMPLICOR HIV Monitor test (Roche Diagnostic Systems Inc., Branchburg, NJ) [Mulder et al., 1994]. Samples were frozen at −80°C within 6 hours of collection and were not thawed until used for testing. One sample from each patient per year was tested using the two techniques.

AMPLICOR HIV Monitor Test

This technique is based on reverse transcription of the RNA corresponding to the HIV *gag* gene and then amplification of the cDNA by PCR.

The RNA quantification using the AMPLICOR test was performed according to the manufacturer's instructions. Briefly, aliquots of 200 μl of serum were treated with lysis solution (guanidium thiocyanate) to release RNA in the presence of a quantitation standard, and the RNA was precipitated by the addition of isopropanol and ethanol. A single enzyme, rT*th*, was used for both reverse transcription and amplification in the presence of biotinylated primers. The resulting biotinylated amplicons were serially diluted (five dilutions) and tested for hybridization with probes for HIV sequences and for the quantitation standard coated onto wells in microtiter plates. Hybridizing amplicons

TABLE I. Quantification of HIV RNA by the Two Techniques

	Techniques	
	AMPLICOR	NASBA
Number of tested samples	106	106
Non-progressor group	53	53
Progressor group	53	53
Number of positives samples (%)	105 (99)	69 (65)
Non-progressor group	52 (98)	24 (45)
Progressor group	53 (100)	45 (85)
HIV RNA titers (log_{10} copies/ml)		
Median	3.59	3.95
Non-progressor group		
Median	3.46	3.63
Range	<2.30–4.33	<3.60–4.76
Progressor group		
Median	4.11	4.36
Range	2.62–5.70	<3.60–5.93

were detected by the addition of avidin-conjugated peroxidase and the chromogenic substrate. The optical density was read at 450 nm, and the titer was calculated as the ratio of HIV signal to the standard signal at the lowest positive dilution. The detection limit of the technique was variable, depending on the efficacy of the amplification, and was generally 2.3 log_{10} HIV RNA copies/ml.

NASBA HIV-1 RNA QT Test

This technique is based on the specific isothermal amplification of RNA corresponding to the HIV *gag* gene involving the synthesis of intermediary DNA using AMV reverse transcriptase, RNase H, and T7 RNA polymerase.

The RNA quantification using the NASBA test was performed according to the manufacturer's instructions. Briefly, aliquots of 100 μl of plasma were treated with lysis solution (guanidium thiocyanate and Triton X-100) containing three internal standards at different concentrations. The RNA was adsorbed onto silica particles which were then washed. The RNA was then released from the silica. A mixture of the primers, dNTP and rNTP was added, the samples were incubated briefly at 65°C, and the enzyme solution was added. The sample was incubated at 41°C for 90 minutes to allow amplification. The amplification products of the patient HIV isolate and the standards were then quantified following capture by a common probe, by hybridization with specific probes labeled with ruthenium (electrochemical luminescence). The concentration of the tested HIV RNA was then calculated as the ratio of the values for the three standards to that of the tested HIV RNA. The detection limit of this technique varied between 3.6 and 4.1 log_{10} HIV RNA copies/ml according to the efficacy of the amplification.

Statistical Analysis

Logarithms of the genome titers were used for statistical analyses. Negative samples were attributed a value of half the detection limit value for the technique. However, for analysis of changes between samples collected on successive years from the same subject, negative samples were attributed the detection limit value for the technique so as not to artificially increase apparent differences.

Agreement between the two techniques was assessed using the K coefficient [Fermanian, 1984] and defining three classes of viral load: <3.3 log_{10}; 3.3 log_{10} to <4 log_{10} and ⩾4 log_{10} HIV RNA copies/ml. The Spearman test was used to compare the genomes titers obtained with the two techniques and to determine the relation of the genome titers with the CD4 lymphocyte counts. The Wilcoxon test was used to compare the genome titers measured by the two techniques and the evolution of genome titers with time in progressors and non-progressors.

ANOVA was used to identify the influence of the progressor and non-progressor groups on the relationship between the viral load and the CD4 lymphocyte count. The evolution of genome titers with time was determined by linear regression. The titer was defined as stable if the slope of the titer plotted against time was not significantly different from 0. The mean genome titer for the non-progressors was determined from all the values measured in each patient except the first one (to avoid the effects of any recent seroconversion, possible with the inclusion criteria used).

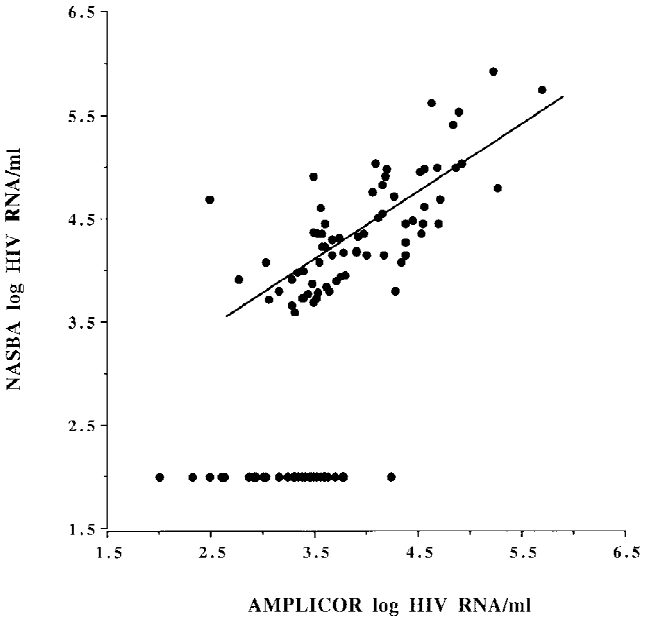

Fig. 1. Comparison of HIV RNA titers obtained by AMPLICOR and NASBA. The AMPLICOR and NASBA techniques were used to test 106 samples. Negative values were excluded for determinations of linear regression between techniques and are represented here as ⩽2 log_{10} RNA copies/ml. The NASBA titers are significantly higher than the AMPLICOR titers ($P \leqslant .0001$, Wilcoxon test); the median difference is 0.41 log_{10} RNA copies/ml. The relationship between the two series of titers is $y = 0.624\ X + 1.922$, $R^2 = 0.525$.

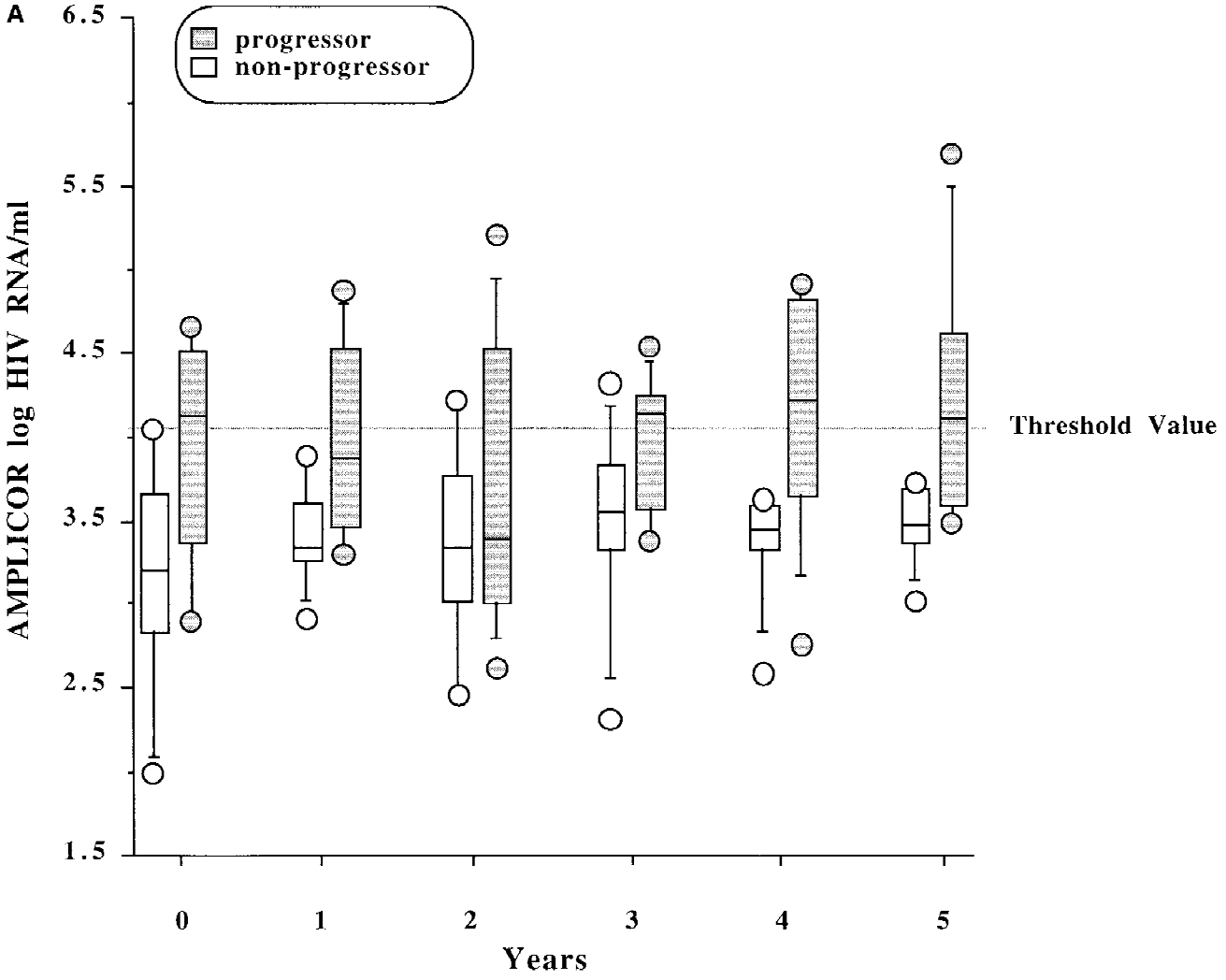

Fig. 2. Comparison of the HIV RNA titers between progressors and non-progressors as measured by AMPLICOR **(A)** and NASBA **(B).** The progressors had significantly higher titers than the non-progressors for years 0, 1, 3, 4, and 5 as determined by AMPLICOR (respectively, $P \leq .05$, $P \leq .06$, $P \leq .04$, $P \leq .03$, $P \leq .02$), and for years 1, 4, and 5 (respectively, $P \leq .03$, $P \leq .07$, and $P \leq .02$) as determined by NASBA. Horizontal bars of boxes indicate medians, the extremities the 75th and 25th percentiles. Horizontal bars outside boxes indicate the 90th and 10th percentiles. Values beyond the 90th and 10th percentiles are shown as circles. The horizontal line indicates the threshold value.

RESULTS

Quantification of HIV RNA by the Two Techniques

One hundred five of the 106 samples (99%) were scored as positive by AMPLICOR and 69 of 106 (65%) by NASBA (Table I).

For the non-progressor group, one of the 53 samples (2%) gave a titer below 2.3 $\log_{10}$ HIV RNA copies/ml (the detection limit) in the AMPLICOR test; 29 of 53 (55%) scored below the detection limit (3.6 to 4.1 $\log_{10}$/ml) in the NASBA technique. For the progressor group, 15% were below the detection limit in the NASBA (Table I).

The results with the two techniques agreed and correlated (concordance coefficient, K, 0.31; Spearman test, $r = 0.73$, $P \leq .0001$).

There were, nevertheless, differences between the positive findings of the two techniques. The titers reported by the NASBA technique were higher than those reported by AMPLICOR (median difference was 0.41 $\log_{10}$ RNA copies/ml, $P \leq .0001$, Wilcoxon test), (Fig. 1). Differences over 0.5 $\log_{10}$ RNA copies/ml (generally considered as being significant) were obtained for 41% of samples. For 9% of the samples, differences over 1 $\log_{10}$ RNA copies/ml were obtained.

Values obtained for successive samples with the same technique were compared. Changes of more than 0.5 $\log_{10}$ were observed by AMPLICOR 28 times (total 86 successive sample pairs), of which 17 were increases and 11 decreases. The corresponding values for the NASBA technique were 31 (total 86), 20, and 11. The two techniques agreed on changes of more than 0.5 $\log_{10}$ RNA copies/ml for 13 pairs of samples.

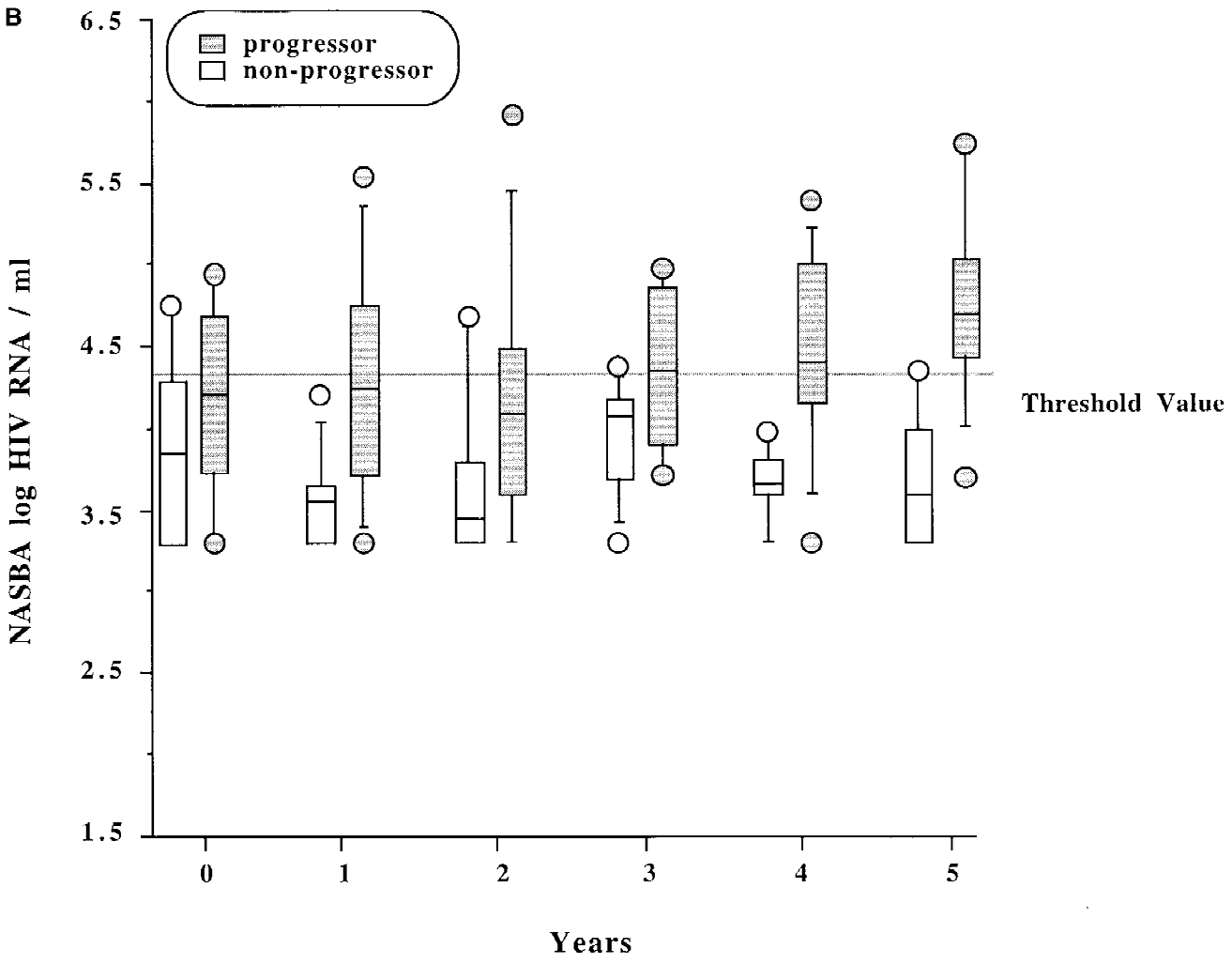

Fig. 2. (continued)

Evolution of Genome Titers in Progressors and Non-Progressors

Overall, the HIV RNA titer obtained in progressors by AMPLICOR was significantly higher than that in non-progressors after 0, 1, 3, 4, and 5 years (Wilcoxon test respectively, $P \leqslant .05$, $P \leqslant .06$, $P \leqslant .04$, $P \leqslant .03$, $P \leqslant .02$) (Fig. 2A). The difference between the two groups was between 0.38 and 0.80 $\log_{10}$ RNA copies/ml. The NASBA test gave similar results after 1, 4, and 5 years (respectively, $P \leqslant .03$, $P \leqslant .07$, $P \leqslant .02$) (Fig. 2B).

The titers for each non-progressor obtained by each technique showed no significant increase during the study (Wilcoxon test and linear regression). A mean titer was therefore calculated for each of these patients for each technique after exclusion of the negative values. The mean of these means was 3.42 $\log_{10}$ RNA copies/ml for AMPLICOR and 4.11 $\log_{10}$ RNA copies/ml for NASBA. A threshold value was defined as the mean plus two standard deviations for this group for each technique: 4.04 $\log_{10}$ RNA copies/ml for AMPLICOR and 4.35 for NASBA. Among the samples from this group, three of 53 scored above this value by AMPLICOR (6%) and five of 53 (9%) by NASBA. No non-progressor scored above this value in two successive tests.

The findings for each patient in the progressor group fell into one of three profiles (Fig. 3). For the first (profile 1, Fig. 3), the genomic titers were above the threshold value and stable (no significant slope as assessed by linear regression); this subgroup included four of the ten progressors as analyzed by AMPLICOR (mean titer 4.60 $\log_{10}$ RNA copies/ml) and six of the ten by NASBA (mean titer 4.72 $\log_{10}$ RNA copies/ml). Two patients presented profile 2: a significant increase in genomic titers with time from a value initially below to a value above the threshold value. This profile was only observed with the AMPLICOR technique. Four patients presented profile 3: stable values below the threshold value throughout the study (mean 3.48 $\log_{10}$ RNA copies/ml by AMPLICOR and 3.92 $\log_{10}$ RNA copies/ml by NASBA).

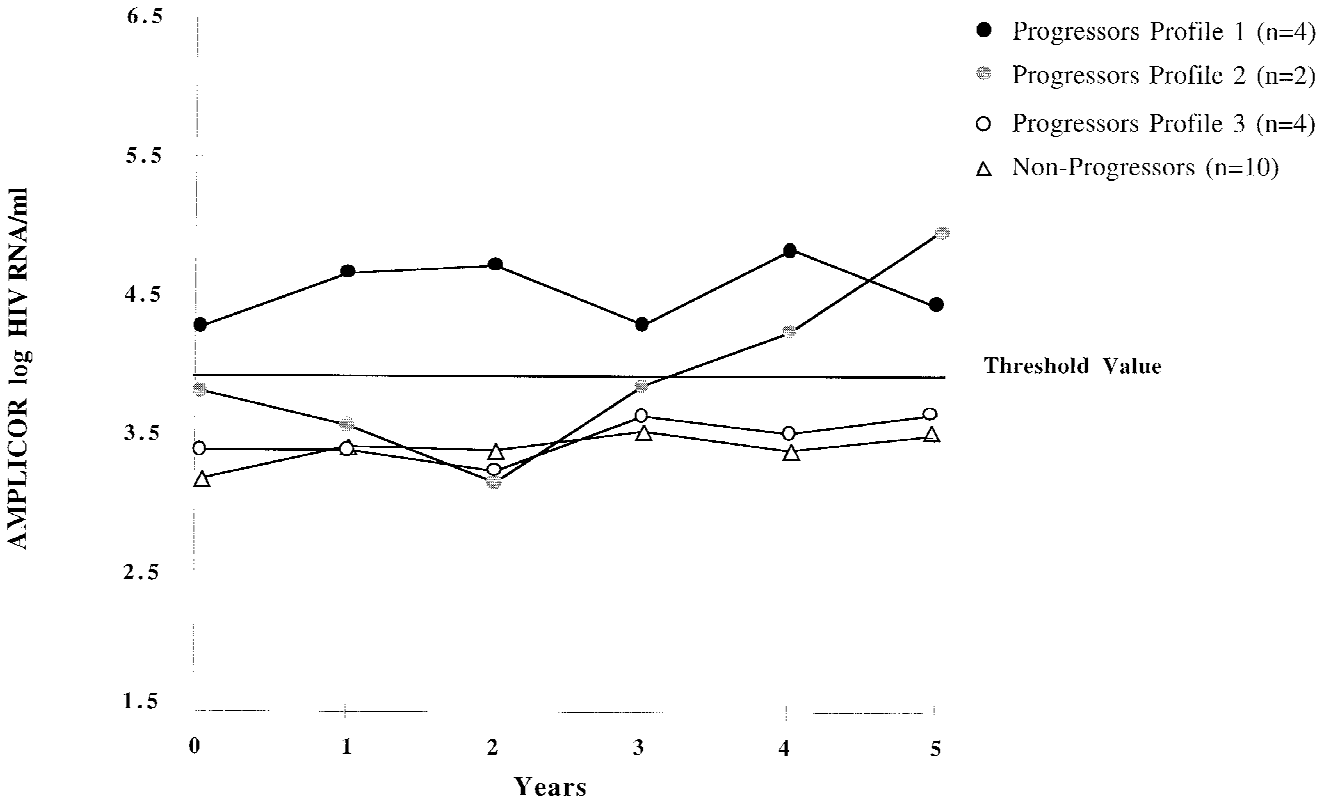

Fig. 3. Profiles of HIV RNA titer changes through time for progressors and non-progressors followed by AMPLICOR. Triangles: the mean HIV RNA titer for the ten non-progressors for each year. Solid circles: the mean HIV RNA titer for each year for the four progressors with stable titers above the threshold value. Shaded circles: the mean HIV RNA titer for each year for the two patients with titers which increased significantly with time. Open circles: the mean HIV RNA titer for each year for the four progressors with titers stable and below the threshold value. The horizontal line shows the threshold value (mean titer for the non-progressors plus two standard deviations: 4.04 $\log_{10}$ RNA copies/ml as assessed by AMPLICOR).

Relation of HIV RNA Titers With CD4 Lymphocyte Counts

Overall, the HIV RNA titer was inversely correlated with the CD4 lymphocyte count (AMPLICOR: $P \leqslant .001$; NASBA: $P \leqslant .003$, Spearman test) (Fig. 4). The relationship between viral load and the CD4 lymphocyte count was influenced by the group (progressor or non-progressor) (AMPLICOR: $P \leqslant .004$; NASBA: $P \leqslant .01$; ANOVA). The correlation between CD4 count and HIV RNA titers is significant for progressors ($P < .001$, Spearman test) but not for non-progressors ($P = .8$, Spearman test).

Of the six cases for which both AMPLICOR and NASBA identified an increase of 0.5 $\log_{10}$ RNA copies/ml in successive titers, five also showed a decrease in the CD4 lymphocyte count for five patients.

Progressors could be classified into two groups according to the evolution of the CD4 lymphocyte count: intermediate progressors (loss of CD4 lymphocytes at a rate of less than 100 cells/mm^3/year) and rapid progressors (>100 cells/mm^3/year). For four of the five rapid progressors the AMPLICOR titers were above the threshold value from the start and throughout the study (profile 1 in Fig. 3), and for all five the NASBA titers were always above the threshold. In contrast, four of the five intermediate progressors gave titers below the threshold value by both the AMPLICOR and NASBA tests (profile 3 in Fig. 3).

DISCUSSION

Mellors et al. [1996] reported the value of HIV RNA titers for predicting the clinical evolution of the disease, and following this marker is essential for therapeutic and clinical management of HIV-infected persons. To allow this marker to be evaluated accurately, the relative performances of the currently used assays (AMPLICOR, NASBA, and QUANTIPLEX) need to be determined. We did not show the results obtained by QUANTIPLEX HIV RNA test because the detection limit of the test was 4 $\log_{10}$ HIV RNA copies/ml (10,000 copies/ml), and the manufacturer had announced major modifications to the test during the study (they commercialized a new test with a sensitivity of 2.7 $\log_{10}$ HIV RNA copies/ml: 500 copies/ml). In this longitudinal study we show that AMPLICOR and NASBA techniques agree satisfactorily. This is consistent with previous transversal studies [Lin et al., 1994; Revets et al., 1996; Vandamme et al., 1996]. Nevertheless, the findings of the techniques differed by more than 0.5 $\log_{10}$ for 41% of the samples. The differences may be due in

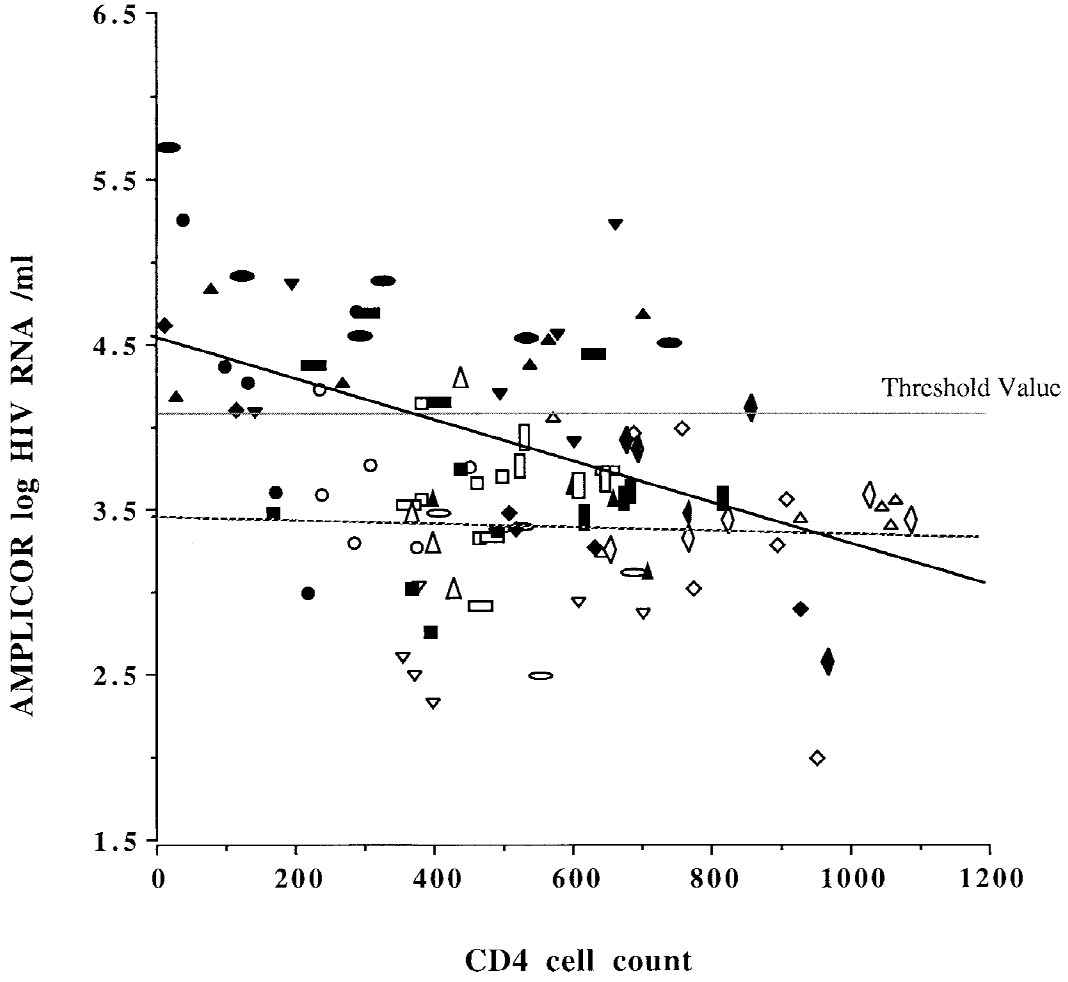

Fig. 4. Correlation between titers obtained by AMPLICOR and the CD4 lymphocyte count. Titers for progressors and non-progressors are shown with solid and open symbols, respectively. For each pair, a different type of symbol is used. The horizontal gray line shows the threshold value. The regression slopes between CD4 count and HIV RNA titers are shown by plain line for progressors and dotted line for non-progressors. The correlation is significant for progressors ($P < .001$, Spearman test) but not for non-progressors ($P = .8$, Spearman test).

part to the use of serum for the AMPLICOR test and plasma for the NASBA test [Holodniy et al., 1995]. Besides the problem of plasma vs. serum, quantification is probably different between the two techniques due to different calibration methods: AMPLICOR was calibrated with nucleic acid standards [Mulder et al., 1994], whereas NASBA used viral suspensions measured by electronic microscopy [Van Gemen et al., 1994]. On the other hand mutations in probe or primer regions cannot explain this difference because this difference is homogeneous among patients.

Consequently, as a difference of 0.5 $\log_{10}$ RNA copies/ml is generally considered significant [Saag et al., 1996], it would be advisable to use the same technique and the same type of sample for each individual patient. The choice of technique involves weighing various advantages and disadvantages: extraction time, sample volume, detection limit, and the number of samples to be tested.

In our study, AMPLICOR or NASBA each frequently (15%) identified separately a change in the titer of more than 0.5 $\log_{10}$ RNA copies/ml between two examinations. These observations may have been due to variability in the tests, and this would suggest that the accepted threshold value for significant change (0.5 $\log_{10}$ RNA copies/ml) is debatable. The threshold was determined by adding the interassay variability to the biological variability [Saag et al., 1996; Winter et al., 1993]. However, the experiments used to determine test variability were performed at one time with the same batches of reagents. In our study the tests were carried out at different times and with different reagents. Indeed, larger variations were reported by Henrard et al. [1995] and Katzenstein et al. [1996], confirming the need to improve the reliability of these techniques. The viral load remained remarkable stable over the study period of 5 years in most of the patients (18 of 20) as assessed by each of the two techniques. This is consistent with previous findings [Henrard et al., 1995; Hogervorst et al., 1995; Katzenstein et al., 1996]. Katzenstein et al. [1996] reported that the titer remained stable until an ascension phase. However, we

did not observe any such increase. The difference could be due to the different populations studied: Two-thirds of the group studied by Katzenstein presented a symptomatic primary infection, and half rapidly developed AIDS. We studied asymptomatic patients, for whom the date of infection was unknown. This type of population is more representative of that encountered in general practice. The stability of HIV RNA titers is the result of a dynamic equilibrium between production of the virus and clearance by the immune system. Approximately 10^9 viruses may be produced and destroyed daily during advanced stages of the disease [Ho et al., 1995; Perelson et al., 1996; Wei et al., 1995]. The stability of the titer is maintained either by the action of cytotoxic T lymphocytes or by the availability of infectable cells [Coffin, 1995].

Although we only studied 20 patients, it was demonstrated that the HIV RNA titer can be used as a predictor of a decline in the CD4 lymphocyte count. This shows the value for this type of analysis of case-control studies as part of prospective surveys particularly to reduce the number of patients that have to be tested. The cases and controls were matched for markers known to be predictive of disease progression: the CD4 lymphocyte count [Shi et al., 1996] and age [Rosenberg et al., 1994]. The predictive value was apparent using the AMPLICOR and NASBA techniques, and AMPLICOR was able to give predictive information earlier. The homogeneity and stability of the RNA titers in the non-progressor group (despite their differing CD4 lymphocyte counts) enabled us to determine a prognostic threshold value, separating the non-progressors from rapid progressors. The value calculated was similar for the two techniques, 4.04 or 4.35 $\log_{10}$ RNA copies/ml, confirming the general concordance between the techniques. This threshold is similar to, and thus confirms, those previously reported [Henrard et al., 1995; Mellors et al., 1995]. The interest of our study is to determine this value using the two techniques on the same samples. Using this threshold value, we could describe three profiles for the evolution of the viral load. Only intermediate progressors had stable RNA titers below the threshold value, when rapid progressors had stable titers above the threshold prior to the decline in the CD4 lymphocyte count. These findings are consistent with those reported for a transversal study [Mellors et al., 1995]. Thus, a high viral load appears to be a clear indication for therapy, independent of the CD4 lymphocyte count. Four of our patients showed low viral loads despite falling CD4 lymphocyte counts. Thus, RNA titers would not have indicated treatment for these patients, although, according to the CD4 count decline, treatment should be discussed. It seems, therefore, that the decision to treat is straightforward when the RNA titer is above 4 $\log_{10}$ RNA copies/ml, but that when the titer is below this value, the CD4 lymphocyte count decline must be considered.

Our study confirms the predictive value of determining the HIV RNA titer in the plasma and shows that it is important for each individual patient to include HIV RNA determinations.

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